

# Comprehensive Investigation of Marine *Actinobacteria* Associated with the Sponge *Halichondria panicea*<sup>†</sup>

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Representatives of *Actinobacteria* were isolated from the marine sponge *Halichondria panicea* collected from the Baltic Sea (Germany). For the first time, a comprehensive investigation was performed with regard to phylogenetic strain identification, secondary metabolite profiling, bioactivity determination, and genetic exploration of biosynthetic genes, especially concerning the relationships of the abundance of biosynthesis gene fragments to the number and diversity of produced secondary metabolites. All strains were phylogenetically identified by 16S rRNA gene sequence analyses and were found to belong to the genera *Actinoballoteichus*, *Micrococcus*, *Micromonospora*, *Nocardiopsis*, and *Streptomyces*. Secondary metabolite profiles of 46 actinobacterial strains were evaluated, 122 different substances were identified, and 88 so far unidentified compounds were detected. The extracts from most of the cultures showed biological activities. In addition, the presence of biosynthesis genes encoding polyketide synthases (PKSs) and nonribosomal peptide synthetases (NRPSs) in 30 strains was established. It was shown that strains in which either PKS or NRPS genes were identified produced a significantly higher number of metabolites and exhibited a larger number of unidentified, possibly new metabolites than other strains. Therefore, the presence of PKS and NRPS genes is a good indicator for the selection of strains to isolate new natural products.

Sponges are multicellular invertebrates and sessile filter feeders which are abundant in the oceans as well as in freshwater habitats (41). They gained great interest due to their association with a wide variety of microorganisms. These microorganisms are known to be a rich source of secondary metabolites (108), which exhibit a broad range of bioactivities such as inhibition of enzyme activities and cell division and antiviral, antimicrobial, anti-inflammatory, antitumor, cytotoxic, and cardiovascular properties (77).

Numerous studies concerning specific aspects of sponge-bacterium associations were accomplished using distinct methods for the evaluation of the microbial diversity (mostly molecular approaches) or the bioactivities (culture-dependent methods) or biosynthetic aspects (chemical analyses and molecular approaches) of secondary metabolites of the associated bacteria (19, 47, 51, 54, 110, 122, 126). So far, there is less comprehensive information about the integration of this knowledge into concepts for sponge-bacterium interactions based on small molecules.

We focused on *Actinobacteria* associated with *Halichondria panicea* Pallas (Porifera, Demospongiae, Halichondriida, Halichondriidae), a sponge species living in coastal habitats worldwide (9). Previous work demonstrated a phylogenetically diverse array of bacterial groups present in this sponge: representatives of *Alphaproteobacteria*, *Betaproteobacteria*, *Gammaproteobacteria*, *Cytophaga/Flavobacteria*, the *Deinococcus*

group, low-G+C-content Gram-positive bacteria, *Actinobacteria*, and *Planctomycetales* were identified by means of a genetic approach (47, 122). Among these, though representing only 3 to 20% of the sponge-associated bacterial community (41, 47, 103), *Actinobacteria* are the most promising bacterial group regarding secondary metabolite production. Members of this phylum account for approximately half of the bioactive secondary metabolites that have so far been discovered in bacteria (64). Although the majority of secondary metabolite-producing *Actinobacteria* originate from terrestrial habitats (101), recent studies of marine *Actinobacteria* have revealed many new chemical entities and bioactive metabolites (13, 30, 50, 100). Among these, only a few substances were isolated from *Actinobacteria* associated with *H. panicea* (85, 123), e.g., the antimicrobially active substances 2,4,4'-trichloro-2'-hydroxydiphenylether and acyl-1-(acyl-6'-mannobiosyl)-3-glycerol produced by *Micrococcus luteus* (17). By combining data about the phylogenetic characterization of the *Actinobacteria* associated with *H. panicea*, their biosynthetic potential for secondary metabolite production, and their chemical profiles, we present comprehensive insights into a great variety of produced natural products as well as their bioactivities. By means of these results, we attempt to close the gap of knowledge about *Actinobacteria* associated with *H. panicea* and discuss the biological roles of identified small molecules in the sponge-associated community.

## MATERIALS AND METHODS

**Collection of sponges.** Living specimens of *H. panicea* Pallas (Porifera, Demospongiae, Halichondriida, Halichondriidae) used in this study were collected several times from November 2002 to June 2004 at the Kiel Fjord, Baltic Sea (Germany), by scuba diving. Additionally, samples of *H. panicea* for the investigation of the metabolite pattern were collected in October 2009 at the Kiel Fjord.

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**Isolation and identification of antimicrobially active *Actinobacteria*.** Approximately 1 g of sponge material was macerated in sterilized habitat water, and the suspension was plated onto five different media according to the method of Wiese et al. (124). Pure cultures were obtained by subsequent plating steps on tryptic soy broth (TSB) medium A (with 10 g/liter NaCl). Incubation was at 22°C in the dark. For storage, pure cultures were kept at -80°C using the Cryobank System according to the instructions of the manufacturer (Mast Diagnostica GmbH, Reinfeld, Germany). Antibiotic activities of the living cultures were determined by an overlay assay as described by Wiese et al. (124) using *Bacillus subtilis* (DSM 347), *Escherichia coli* K-12 (DSM 498), *Staphylococcus lentus* (DSM 6672), and *Candida glabrata* (DSM 6425) as test strains. Antibiotically active strains were selected for the present study. To identify all strains, 16S rRNA gene sequence analyses were carried out according to the protocol of Thiel et al. (110). Comparison of the 16S rRNA gene sequences of the sponge-derived strains was performed using the Basic Local Alignment Search Tool (nucleotide blast) with the EMBL nucleotide database available at the European Bioinformatics Institute website (<http://www.ebi.ac.uk/>) (2) and the Ribosomal Database Project II (RDP-II) website (21).

**Identification of NRPS and PKS-II gene fragments.** All bacterial strains were screened for genes corresponding to the nonribosomal peptide synthetase (NRPS) adenylation domain and the polyketide synthase type II (PKS-II) KS<sub>α</sub> domain by the methods developed by Doekel and Marahiel and Metsä-Ketelä et al. (24, 73). The PCR products had expected sizes of 250 to 270 bp (for NRPS gene fragments) and 580 to 620 bp (for PKS gene fragments). Sequences of these PCR products were compared with NRPS and PKS sequences in the EMBL nucleotide database available at the European Bioinformatics Institute website by using the Basic Local Alignment Search Tool (blastx) (2).

**Cultivation of liquid cultures.** For the analyses of secondary metabolite profiles, bacteria were grown in 300-ml Erlenmeyer flasks each containing 100 ml of medium GYM4 (10 g glucose, 4 g yeast extract, 4 g malt extract, 1 liter water, pH 7.2) for 5 days at 28°C with shaking (at 120 rpm) in the dark. Each culture was inoculated separately with a 1-cm<sup>2</sup> piece from a culture grown on a GYM4 agar plate for 4 weeks at 28°C in the dark.

**Extraction of liquid cultures.** The entire culture broth (100 ml) was extracted by homogenization with 50 ml ethyl acetate by using an Ultra-Turrax T25 basic disperser (IKA-Werke GmbH and Co., Staufen, Germany) at 13,000 rpm for 30 s. The supernatants were separated, dried, and subsequently resolved in methanol. These crude extracts were used for antimicrobial assays and chemical analyses.

**Extraction of sponge specimens.** Approximately 100 g wet sponge tissue was extracted by homogenization with 500 ml methanol by using an Ultra-Turrax T25 basic disperser (IKA-Werke GmbH and Co., Staufen, Germany) at 13,000 rpm. Afterwards the homogenate was stirred overnight at room temperature. The resulting supernatant was separated from the cell residue, dried *in vacuo*, and subsequently resolved in 5 ml methanol. These crude extracts were used for the chemical analysis.

**Antimicrobial activities of culture extracts.** Antimicrobial assays were performed with the following test organisms: *B. subtilis* (DSM 347), *E. coli* K-12 (DSM 498), *S. lentus* (DSM 6672), *Pseudomonas syringae* pv. aptata (DSM 50252), *Pseudomonas fluorescens* (NCIMB 10586), *Xanthomonas campestris* (DSM 2405), *Ralstonia solanacearum* (DSM 9544), *C. glabrata* (DSM 6425), and *Erwinia amylovora* (DSM 50901). Assay mixtures were prepared by transferring 10-μl aliquots of methanolic solutions of extracts or pure substances into two wells of a 96-well microtiter plate and evaporating the solvent in a vacuum centrifuge. Overnight cultures of the test organisms in TSB medium (tryptic soy broth [Difco], 12 g/liter; NaCl, 5 g/liter) were diluted to an optical density at 600 nm (OD<sub>600</sub>) of 0.02 to 0.05, and 200-μl aliquots of the resulting suspensions were added to the wells. After incubation for 15 h at 600 rpm and 28°C, 10 μl of a resazurin solution (0.2 mg/ml phosphate-buffered saline) was added to each well and the plate was incubated at 28°C until the color of the negative control changed from blue to pink. For evaluation of the cell viability, the transformation of resazurin to resorufin was assayed by measuring the fluorescence at 560 nm after excitation at 590 nm. The resulting values were compared to those for a positive control (100 μg/well chloramphenicol for bacteria and 200 μg/well cycloheximide for *Candida*) and a negative control (no compound) on the same plate.

**Chemical analyses of culture extracts and sponge extract.** Dereplication of substances was realized by comparison of mass spectrometry (MS) and UV data obtained by high-performance liquid chromatography (HPLC)-UV/MS analyses. Reversed-phase HPLC experiments were performed by using a Phenomenex Onyx Monolithic C<sub>18</sub> column (100 by 3.00 mm) and applying an H<sub>2</sub>O (solvent A)-acetonitrile (MeCN; solvent B) gradient with 0.1% HCOOH added to both solvents (gradient program: 0 min, 5% solvent B; 4 min, 60% solvent B; 6 min,

100% solvent B; flow, 2 ml/min) on a VWR Hitachi Elite LaChrom system coupled to an electrospray ionization (ESI) ion trap detector (Esquire 4000; Bruker Daltonics). The resulting chemical data were compared with data from the Antibase (63) and the Chapman & Hall/CRC Chemical Database *Dictionary of Natural Products* (15). Several extracts were subjected to preparative HPLC with a Phenomenex Gemini-NX C<sub>18</sub> column (5 μm, 110 Å, Axia packed; 100 by 21.20 mm) and variable gradients of eluents H<sub>2</sub>O (solvent A) and MeCN (solvent B). For selected substances, a <sup>1</sup>H nuclear magnetic resonance (NMR) analysis was carried out. NMR spectra were recorded on a Bruker AV 600 spectrometer (at 600 and 150 MHz) by using the signals of the residual solvent protons as internal references (chemical shift δH 3.31 ppm for protons and δC 49.0 ppm for carbons; NMR solvent, deuterium-labeled methanol [MeOH-D<sub>4</sub>]). High-resolution mass spectra were acquired on a benchtop time-of-flight spectrometer (MicroTOF; Bruker Daltonics) with positive ESI.

**Nucleotide sequence accession numbers.** The nucleotide sequence data reported in the present study were deposited in the GenBank nucleotide sequence database under the accession numbers GQ863900 to GQ863929, GQ863931 to GQ863933, GU213489 to GU213493, and GU213495 to GU213502 (for 16S rRNA sequences), GQ863946 to GQ863965 (for NRPS sequences), and GQ863934 to GQ863945 and GU320045 (for PKS sequences).

## RESULTS

**Isolation and identification of *Actinobacteria* from *H. panicea*.** Among a large number of bacteria isolated from the sponge *H. panicea*, 46 isolates were classified as *Actinobacteria* belonging to the genera *Actinoballoteichus*, *Micrococcus*, *Micromonospora*, *Nocardiosis*, and *Streptomyces*, with members of the last genus, comprising 39 strains, being the most abundant (Table 1).

**Identification of NRPS and PKS-II gene fragments.** All actinobacterial strains were analyzed for the presence of PKS-II and NRPS genes (Table 1; see also the supplemental material). In 16 strains (35%), neither NRPS nor PKS gene fragments were detected. An NRPS PCR product of the expected size was recovered from 24 strains (52%), and 20 of these products had sequences similar (40 to 96%) to known NRPS gene fragments. These similarities give basic information on the presence of NRPS genes, but correlation to specific metabolites is limited due to the lengths of the PCR fragments. The PCR products of four strains showed more than one sequence with similar lengths in the electropherogram, indicating multiple NRPS gene clusters.

PKS sequences with similarities to known PKS gene fragments were obtained from 13 (28%) of the *Actinobacteria* strains (see the supplemental material). Similarities between the gene sequences of the investigated *Actinobacteria* and known sequences ranged from 47% (for a sequence from strain HB328 and the ketosynthase gene sequence from *Streptomyces felleus* BAF43343) to 100% (for a sequence from strain HB202 and a PKS sequence from *Streptomyces* sp. BAH68110). For 7 strains (15%), PCR products for both PKS-II and NRPS genes were detected.

**Chemical analyses of culture extracts.** The *Actinobacteria* strains isolated from *H. panicea* turned out to be potent producers of secondary metabolites, with 40 of 46 strains producing detectable amounts of metabolites. In total, 122 different substances were identified from the secondary metabolite patterns of the analyzed *Actinobacteria* strains by using HPLC-MS, UV, and NMR techniques (Tables 2 and 3). The numbers and diversity of produced compounds indicated by the metabolite profiles were evaluated with respect to the presence of PKS and NRPS biosynthetic genes. A much higher level of

TABLE 1. *Actinobacteria* from *H. panicea*: identification, detection of PKS-II and NRPS gene fragments, and chemical analyses

Strain	Most closely related type strain <sup>a</sup>	16S rRNA gene sequence accession no. <sup>b</sup>	Gene fragment(s) identified	Chemically identified product(s) <sup>c</sup>	Detection of new compound(s) <sup>d</sup>
HB062	<i>Streptomyces griseus</i> ATCC 25497	D63872	PKS and NRPS fragments	Other	X
HB084	<i>Streptomyces intermedius</i> DSM 40372	Z76686		Other	
HB094	<i>Streptomyces sampsonii</i> ATCC 25495	D63871			
HB095	<i>Streptomyces rugosus</i> DSM 40077	Z76688		Other	
HB096	<i>Streptomyces rugosus</i> DSM 40077	Z76688		Other	
HB099	<i>Streptomyces griseus</i> ATCC 25497	D63872			X
HB100	<i>Streptomyces platensis</i> JCM 4662	AB045882	PKS and NRPS fragments	Other	
HB101	<i>Streptomyces libani</i> subsp. <i>rufus</i> LMG 20087	AJ781351	NRPS fragment	Pep	
HB102	<i>Streptomyces scabiei</i> ATCC 49173	D63862	NRPS fragment	Other	
HB105	<i>Streptomyces sampsonii</i> ATCC 25495	D63871		Other	
HB107	<i>Streptomyces platensis</i> JCM 4662	AB045882	NRPS fragment	Other, pep	X
HB108	<i>Streptomyces fulvorozeus</i> LMG 19901	AJ781331			
HB110	<i>Streptomyces griseus</i> ATCC 25497	D63872			
HB113	<i>Streptomyces fulvorozeus</i> LMG 19901	AJ781331	PKS and NRPS fragments <sup>e</sup>	Other, pep	X
HB114	<i>Streptomyces griseus</i> ATCC 25497	AF112160			X
HB115	<i>Streptomyces mediolani</i> LMG 20093	AJ781354			
HB116	<i>Streptomyces sampsonii</i> ATCC 25495	D63871		Other, arom pk	
HB117	<i>Streptomyces fulvorozeus</i> LMG 19901	AJ781331	PKS fragment	Other	
HB118	<i>Streptomyces sampsonii</i> ATCC 25495	D63871		Other, arom pk	
HB122	<i>Streptomyces luridiscabiei</i> S63	AF361784		Other	
HB132	<i>Streptomyces atroolivaceus</i> LMG 19306	AJ781320	PKS and NRPS fragments <sup>e</sup>	Other	X
HB138	<i>Streptomyces fulvorozeus</i> LMG 19901	AJ781331	NRPS fragment	Other, pep	X
HB140	<i>Streptomyces griseus</i> ATCC 25497	D63872	NRPS fragment	Other, pep	
HB142	<i>Streptomyces intermedius</i> DSM 40372	Z76686	NRPS fragment	Other	X
HB149	<i>Streptomyces griseus</i> JCM 4623	AB045872	NRPS fragment		X
HB156	<i>Streptomyces koyangensis</i> VK-A60	AY079156	NRPS fragment	Other	
HB157	<i>Streptomyces sampsonii</i> ATCC 25495	D63871	NRPS fragment	Other, arom pk	
HB181	<i>Streptomyces fulvorozeus</i> LMG 19901	AJ781331	NRPS fragment	Other, pep	X
HB184	<i>Streptomyces mediolani</i> LMG 20093	AJ781354		Other	
HB200	<i>Streptomyces pulveraceus</i> LMG 20322	AJ781377	PKS and NRPS fragments <sup>e</sup>	Other	X
HB202	<i>Streptomyces mediolani</i> LMG 20093	AJ781354	PKS and NRPS fragments	Other	X
HB238	<i>Micromonospora matsumotoense</i> IMSNU 22003	AF152109	NRPS	Pep <sup>f</sup>	
HB241	<i>Micrococcus luteus</i> ATCC 4698	AF542073		Other	
HB243	<i>Streptomyces mediolani</i> LMG 20093	AJ781354	NRPS fragment	Other, pep	
HB253	<i>Micromonospora matsumotoense</i> IMSNU 22003	AF152109	NRPS fragment <sup>e</sup>		X
HB254	<i>Micromonospora matsumotoense</i> IMSNU 22003	AF152109			X
HB272	<i>Actinobolus cyanogriseus</i> C7654	AY094367	NRPS fragment	Other, pep	X
HB274	<i>Streptomyces platensis</i> JCM 4662	AB045882	NRPS fragment		X
HB288	<i>Streptomyces odorifer</i> DSM 40347	Z76682	NRPS fragment	Other, pep	
HB291	<i>Streptomyces fulvorozeus</i> LMG 19901	AJ781331	PKS and NRPS fragments	Other	X
HB298	<i>Streptomyces coelestis</i> ICSSB 1021	AF503496	PKS fragment	Other	
HB320	<i>Streptomyces sanglieri</i> A5843	AY094363	PKS fragment	Other, arom pk	X
HB328	<i>Streptomyces fulvorozeus</i> LMG 19901	AJ781331	PKS fragment	Other	
HB375	<i>Micromonospora aurantiaca</i> DSM 43813	X92604	NRPS fragment	Other	X
HB381	<i>Streptomyces griseus</i> ATCC 25497	D63872	PKS fragment		
HB383	<i>Nocardopsis alba</i> DSM 43377	X97883	PKS fragment		X

<sup>a</sup> The analyzed sequences from each strain and the corresponding type strain had a level of similarity greater than or equal to 98.5%.

<sup>b</sup> NCBI accession number.

<sup>c</sup> Pep, peptide; arom pk, aromatic polyketide; other, secondary metabolite(s) other than a peptide or aromatic polyketide. Identified compounds are listed in Tables 2 and 3.

<sup>d</sup> X indicates the occurrence of a new compound.

<sup>e</sup> A PCR product(s) in the expected size range was obtained. Affiliation of the obtained sequence to known genes was not possible.

<sup>f</sup> Proven NRPS biosynthetic pathway.

diversity of natural products was found in strains with proof of PKS or NRPS genes than in those with negative PCR results (23 different structural groups versus 7 different structural groups) (Fig. 1). Strains exhibiting no PKS-II or NRPS amplicons synthesized only a small spectrum of compounds, mainly antimycin derivatives. In the 30 *Actinobacteria* strains with positive PCR results for PKS genes (13 strains) and/or NRPS

genes (24 strains), a total of 108 different substances were identified. In only one of these strains, no metabolite could be detected, while 17 strains (57%) produced substances (a total of 88 compounds) that have not been identified so far and are probably new natural products (Table 1). Elucidation of the structures of these compounds is in progress. Among the 16 strains which gave no hints of the presence of PKS-II or NRPS



TABLE 2. Identified metabolites from *H. panicea*-associated actinobacterial strains positive for a PKS and/or NRPS gene fragment by PCR

No.	Source(s)	Compound (reference)	Chemical group	Activity(ies)	Dereplication analyses
1	HB062	6'''-O- $\alpha$ -D-Mannopyranosylmannosido-streptomycin (45)	Aminoglycoside	Antibacterial	MS, UV
2	HB100	Oxazolomycin (53)	$\beta$ -Lactone, polyene	Phytotoxic	MS, UV, NMR
3	HB100	Coproporphyrin (113)	Porphyrin	Zinc chelator, photosensitizer	MS, UV
4	HB101	Pepstatin AC (5)	Peptide	Pepsin inhibitor	MS, UV, NRPS-specific PCR
5	HB101	Pepstatin CU (5)	Peptide	Pepsin inhibitor	MS, UV, NRPS-specific PCR
6	HB102	Clavamycin D (57)	$\beta$ -Lactam antibiotic	Antifungal	MS, UV
7	HB107	4-Thiouracil (25)	Nucleoside analog	Antibacterial, cytotoxic	MS, UV
8	HB107	Bovinocidin (4)	Dipeptide	Antibacterial	MS, UV, NRPS-specific PCR
9	HB107	Trihomononact acid (93)	Lactone	Active against Gram-positive bacteria and brine shrimp	MS, UV
10	HB113, HB272	Antibiotic NA 22598A <sub>1</sub> (62)	Peptide	Cytotoxic	MS, UV, NRPS-specific PCR
11	HB113, HB140	3-O-( $\alpha$ -L-Mycarosyl)erythronolide B (71)	Macrolide	ND <sup>a</sup>	MS, UV
12	HB113	Gualamycin (114)	Aminoglycoside	Acaricidal	MS, UV
13	HB113	Antibiotic X 14931A (120)	Polyether	Active against Gram-positive bacteria, fungi, and protozoa	MS, UV
14	HB113	17-Methylenespiramycin I (68)	Macrolide	ND	MS, UV
15	HB113	Streptomycin (116)	Aminoglycoside	Antibacterial, neuromuscular blocking agent, phospholipase D inhibitor	MS, UV
16	HB113	Antibiotic M-2846 (105)	Macrolide	Active against Gram-positive bacteria	MS, UV
17	HB113	1-Epimanzamine D (127)	Alkaloid	Antibacterial, cytotoxic	MS, UV
18	HB113	Antibiotic A 75943 (75)	$\delta$ -Lactone	Bone resorption inhibitor	MS, UV
19	HB113	N-Didemethylallosamidin (81)	Aminoglycoside	Chitinase inhibitor, insecticide, antifungal	MS, UV
20	HB113	Methylallosamidin (99)	Aminoglycoside	Insect chitinase inhibitor	MS, UV
21	HB113	Allosamidin (99)	Aminoglycoside	Insecticide	MS, UV
22	HB117, HB291	Dihydromaltophilin (34)	Macrolactam	Antifungal	MS, UV
23	HB117, HB181, HB291	Senacarcin A (79)	Phenazine	Antibacterial, cytotoxic	MS, UV
24	HB117, HB328	Pyrocoll (22)	Alkaloid	Antibacterial, antifungal, cytotoxic, antiprotozoan, antiparasitic	MS, UV, NMR
25	HB117, HB291	Streptazone B1 (90)	Alkaloid	Cytotoxic	MS, UV
26	HB132	Venturicidin A (95)	Macrolide	Antifungal	MS, UV
27	HB132	Irumamycin (83)	Lactone	Antifungal	MS, UV
28	HB138	Bundlin A (115)	Macrolide	Active against Gram-positive bacteria and fungi	MS, UV
29	HB138	Neoenactin B1 (98)	Hydroxamic acid	Antifungal	MS, UV
30	HB138	Ashimycin A (112)	Aminoglycoside	Antibacterial	MS, UV
31	HB138	Validamycin D (42)	Aminoglycoside	Antifungal	MS, UV
32	HB138	N-Demethylstreptomycin (31)	Aminoglycoside	Antimicrobial	MS, UV
33	HB138	Detoxin E <sub>1</sub> (84)	Depsipeptide	Cytotoxic	MS, UV
34	HB138	Antibiotic X 14873A (67)	Polyether	Active against Gram-positive bacteria	MS, UV
35	HB138	Valistatin (58)	Tripeptide	Aminopeptidase M inhibitor	MS, UV, NRPS-specific PCR
36	HB140	Phencomycin ethyl ester (91)	Phenazine	Antibacterial, cytotoxic	MS, UV
37	HB140	Pepstatin BU (5)	Peptide	Pepsin inhibitor	MS, UV, NRPS-specific PCR
38	HB140	N-Acetylglucylclavamycin acid (6, 92)	$\beta$ -Lactam	$\beta$ -Lactamase inhibitor	MS, UV
39	HB142, HB156	Isovaleryl-6-deoxy- $\alpha$ -L-talopyranoside (11)	Glycoside	ND	MS, UV
40	HB156	Polyene with 8 or 9 double bonds	Polyene	Antifungal	MS, UV
41	HB156	Galbonolide A (28)	Macrolide	Antifungal	MS, UV
42	HB156, HB291	5-Hydroxy-3-(1-hydroxy-2-methylpropyl)-4-methyl-2(5H)-furanone (14)	Lactone	Antibacterial, chitinase and phosphatase inhibitor	MS, UV
43	HB156	Albaflavonone (36)	Sesquiterpene ketone	Antibacterial	MS, UV
44	HB157	3,4-Dimethyl-7-(methylamino)-5,8-isoquinolinedione (70)	Quinone	Active against Gram-positive bacteria	MS, UV
45	HB157, HB288	5-(6'-Methyl-7'-oxo-octyl)-SH-furan-2-one (76)	Lactone	Cytotoxic	MS, UV, NMR
46	HB157	Polyene with 7 or 8 double bonds	Polyene	Antifungal	MS, UV
47	HB157	Fredericamycin A (117)	Aromatic polyketide	Cytotoxic generator of free radicals	MS, UV
48	HB157, HB181	Antimycin A4 (8)	Macrolide	Inhibitor of ATP-citrate lyase	MS, UV, NMR
49	HB157, HB181, HB288	Antimycin A3 (8)	Macrolide	Cytotoxic, antiviral, inhibitor of ATP-citrate lyase	MS, UV, NMR
50	HB157, HB181, HB288	Antimycin A2 (8)	Macrolide	Inhibitor of ATP-citrate lyase	MS, UV, NMR

Continued on following page

TABLE 2—Continued

No.	Source(s)	Compound (reference)	Chemical group	Activity(ies)	Dereplication analyses
51	HB157, HB181, HB288	Antimycin A1 (8)	Macrolide	Antiviral inhibitor of ATP-citrate lyase, ichthyotoxin, inhibitor of ubiquinol-cytochrome C oxidoreductase	MS, UV, NMR
52	HB157	Antimycin A0 (8)	Macrolide	Antifungal	MS, UV, NMR
53	HB157	Antimycin A5 (8)	Macrolide	ND	MS, UV, NMR
54	HB181	Histidinomycin (48)	Peptide	Antifungal	MS, UV, NRPS-specific PCR
55	HB181	Nebramycin (111)	Aminoglycoside	Antimicrobial	MS, UV
56	HB181, HB243	Antibiotic X 14873G (67)	Polyether	Active against Gram-positive bacteria	MS, UV
57	HB181	Mycospocidin (78)	Nucleoside	Active against Gram-positive bacteria	MS, UV
58	HB181	5-Hydroxy-4-oxonorvaline (52)	Amino acid	Antifungal	MS, UV
59	HB181	3-Formamido-2-hydroxy-N-[(2R,6S,7R,8R)-7-hydroxy-2,6,8-trimethyl-4,9-dioxo-1,5-dioxonan-3-yl]benzamide (37)	Macrolide (antimycin)	ND	MS, UV
60	HB181	N-[(2R,3S,6S,7R,8R)-8-Ethyl-7-hydroxy-2,6-dimethyl-4,9-dioxo-1,5-dioxonan-3-yl]-3-formamido-2-hydroxybenzamide (37)	Macrolide (antimycin)	ND	MS, UV
61	HB181	Blastamycin (37)	Macrolide (antimycin)	Antifungal, inhibitor of ATP-citrate lyase	MS, UV
62	HB181	Kitamycin A (39)	Macrolide (antimycin)	Plant growth inhibitor	MS, UV
63	HB181	5-{3-[(3-Formamido-2-hydroxybenzoyl)amino]-2,6-dimethyl-8-octyl-4,9-dioxo-1,5-dioxonan-7-yl}pentanoic acid (37)	Macrolide (antimycin)	ND	MS, UV
64	HB181	Bafilomycin D (60)	Macrolide	Inhibitor of membrane ATPases, insecticide	MS, UV
65	HB200	Antibiotic BE 10988 (82)	Thiazolyindole	Cytotoxic, topoisomerase II inhibitor	MS, UV, NRPS-specific PCR <sup>b</sup>
66	HB200	N-Methyltyrosyl-N-methyltyrosylleucylalanine (3)	Peptide	ND	MS, UV
67	HB202	Streptophenazin A (74)	Phenazine	Active against Gram-positive bacteria	MS, UV, NMR
68	HB202	Streptophenazin B (74)	Phenazine	Active against Gram-positive bacteria	MS, UV, NMR
69	HB202	Streptophenazin C (74)	Phenazine	Active against Gram-positive bacteria	MS, UV, NMR
70	HB202	Streptophenazin D (74)	Phenazine	Active against Gram-positive bacteria	MS, UV, NMR
71	HB202	Streptophenazin E (74)	Phenazine	Active against Gram-positive bacteria	MS, UV, NMR
72	HB202	Streptophenazin F (74)	Phenazine	Active against Gram-positive bacteria	MS, UV, NMR
73	HB202	Streptophenazin G (74)	Phenazine	Active against Gram-positive bacteria	MS, UV, NMR
74	HB202	Streptophenazin H (74)	Phenazine	Active against Gram-positive bacteria	MS, UV, NMR
75	HB238	Rotihibin B (32)	Peptide	Plant growth regulator	MS, UV, NRPS-specific PCR
76	HB238	Heptakis-(6-deoxy)-cyclomaltoheptose (15)	Glycoside	ND	MS, UV
77	HB238	Antibiotic GAI 3 (15)	Glycoside	ND	MS, UV
78	HB238	Antibiotic GAI 2 (15)	Glycoside	ND	MS, UV
79	HB238	Fortimicin KR <sub>1</sub> (107)	Aminocyclitol	Antibacterial	MS, UV
80	HB238	Massetolide K (26)	Lipopeptide	Antibacterial	MS, UV, NRPS-specific PCR
81	HB243	Sulfostin (1)	Alkaloid	Inhibitor of dipeptidyl peptidase IV	MS, UV
82	HB243	Fosfazinomycin A (35)	Aminophosphonic acid	Antifungal	MS, UV
83	HB243	Tobramycin (59)	Aminoglycoside	Antibacterial	MS, UV
84	HB243	N-Acetylvalylleucylargininal (69)	Peptide	ND	MS, UV, NRPS-specific PCR
85	HB272	Hemipyocyanine (102)	Phenazine	Active against Gram-positive bacteria, antifungal, antiviral, algicide	MS, UV
86	HB272	Daryamide C (7)	Polyketide	Cytotoxic	MS, UV
87	HB272	Antibiotic A 58365B (80)	Nucleoside	Cytotoxic	MS, UV
88	HB272	Antibiotic SW 163B (106)	Octadepsipeptide	Antifungal, immunosuppressant	MS, UV
89	HB274	Dihydro-4-(hydroxymethyl)-3-(3-methylbutanonyl)-2(3H)-furanone (15)	Furanone	ND	MS, UV

Continued on following page

TABLE 2—Continued

No.	Source(s)	Compound (reference)	Chemical group	Activity(ies)	Dereplication analyses
90	HB274	4-Hydroxythreonine (121)	Amino acid	Antibacterial	MS, UV
91	HB288	Albopeptin A (49)	Peptide	Antifungal	MS, UV, NRPS-specific PCR
92	HB288	PD 125375 (96)	Pyrrol, amide	Cytotoxic	MS, UV
93	HB291	Lankamycin (33)	Macrolide	Antibacterial	MS, UV
94	HB298	Germicidin (86)	Pyrone	Retards germination of cress; inhibitor of porcine Na <sup>+</sup> /K <sup>+</sup> -activated ATPase	MS, UV, NMR
95	HB298	Inthomycin A (118)	Polyketide	Antimicrobial, herbicidal	MS, UV, NMR
96	HB298	Inthomycin B or C (40)	Polyketide	ND	MS, UV, NMR
97	HB320	Blanchaquinone (20)	Anthraquinone	Antibacterial, cytotoxic	MS, UV, PKS
98	HB320	Antibiotic MA 1189 (102)	Benzenediol	5-Lipoxygenase inhibitor	MS, UV
99	HB320	Antibiotic K 502A (125)	Anthracycline	ND	MS, UV, PKS-specific PCR
100	HB320	Homo-nonactyl-nonactate (94)	Dilactone	ND	MS, UV
101	HB328	Futalosine (43)	Nucleoside	Cytotoxic	MS, UV
102	HB328	Antibiotic X 14889A (66)	Polyether	Active against Gram-positive bacteria	MS, UV
103	HB328	Mutalomycin (29)	Polyether	Ionophore	MS, UV
104	HB375	Diazepinomicin (18)	Alkaloid	Antimicrobial	MS, UV
105	HB375	Fortimicin AH (27)	Aminocyclitol	Antibacterial	MS, UV
106	HB375	Rakicidin A (72)	Lipopeptide	Hypoxia-selective cytotoxic agent	MS, UV
107	HB375	Rakicidin B (72)	Lipopeptide	Cytotoxic	MS, UV
108	HB375	Polyene with ca. 12 double bonds	Polyene	Antifungal	MS, UV

<sup>a</sup> ND, not determined.<sup>b</sup> A PCR product of the estimated size was obtained. Sequence similarity could not be detected.

genes, 4 strains (25%) did not produce any detectable metabolite, while in the remaining 12 strains, a total of 27 compounds were identified. Only 3 of those 12 metabolite-producing bacteria were capable of the biosynthesis of natural products (a total of 11 compounds) not listed in any database used (Table 1).

The identified substances belong to 23 different structural groups. Most frequent were polyketides (35 compounds), peptides (16 compounds), phenazines (13 compounds), and aminoglycosides (13 compounds). In total, 77 substances belonged to these 4 most prominent structural groups; the other 19 structural groups were represented by only one or a few metabolites (Fig. 1). For example, only strain HB298 produced a single pyrone. Just four glycosides were identified; one of these was heptakis-(6-deoxy)-cyclomaltoheptose, produced by strain HB238.

Although specific prediction of products of NRPS and PKS genes according to the obtained sequences is problematic, because only small gene fragments were amplified, an attempt was made to correlate the structural properties of produced secondary metabolites with the presence of PKS-II and NRPS genes. Even though we expected aromatic polyketides in 13 strains (Table 1) with a matching sequence for a PKS-II gene, aromatic polyketides were identified in only 3 strains. Strain HB320 produced two aromatic polyketides, antibiotic K 502A and blanchaquinone. Strains HB062 and HB202 produced possibly new aromatic polyketides. In 10 of the strains yielding PCR products with the PKS-specific primers, no corresponding PKS products were found under the applied culture conditions. On the other hand, the strains HB116, HB118, and HB157 produced the aromatic polyketide fredericamycin A and strain HB184 produced enterocin, although the presence of PKS-II genes in these strains could not be confirmed. Of 20 *Actinobacteria* strains containing NRPS gene fragments, 11 were shown to produce one or more peptides. For example,

strain HB238 produced massetolide K (Table 2), a known lipopeptide which has been reported to be NRPS derived.

**Chemical analysis of sponge extract.** We were able to detect the substances fosfazinomycin and tobramycin in the methanolic extract from *H. panicea*. These two substances were secondary metabolites identified in strain HB243.

**Antimicrobial activities of culture extracts.** All strains were selected according to their antibiotic activities by an overlay assay. Subsequently, only 31 (67%) of 46 culture extracts showed activity against at least one of the test strains (Table 4). Frequent activities against *B. subtilis* (44% extracts), *S. lentus* (33%), *R. solanacearum* (24%), and *C. glabrata* (26%) were detected. More rarely, activities against *E. coli* (9%), *P. fluorescens* (7%), *P. syringae* (9%), *X. campestris* (15%), and *E. amylovora* (9%) were observed. The extracts revealed 18 different activity patterns ranging from no activity at all to activities against even seven of nine test organisms, as illustrated in Table 4. In 15 crude extracts, we were not able to find any microbial activity. Eight (53%) of these extracts additionally did not show any substance in the chromatogram (Tables 1 and 4). Eleven (73%) of the 15 extracts with no antimicrobial activity did not show any NRPS or PKS gene fragment. Seven (47%) yielded neither antimicrobial activity nor a gene fragment.

**Bioactivities of identified compounds known from literature.** Data on the biological activities of most of the secondary metabolites isolated from *Actinobacteria* and identified via HPLC-UV/MS analyses in this study were compiled by a profound literature search concerning reported biological activities (e.g., antibacterial, antifungal, cytotoxic, immunosuppressive, antiviral, and algicidal). The results are summarized in Tables 2 and 3. In particular, activities against Gram-positive bacteria and fungi as well as cytotoxic activities were described for a number of these metabolites. However, in some extracts, metabolites with exceptional activities, like rothibin B from

TABLE 3. Identified metabolites from *H. panicea*-associated actinobacterial strains negative for a PKS and/or NRPS gene fragment by PCR

No.	Source(s)	Compound (reference)	Chemical group	Activity(ies)	Dereplication analyses
1	HB084, HB095, HB096, HB116, HB118	5-(6'-Methyl-7'-oxo-octyl)-SH-furan-2-one (76)	Lactone	Cytotoxic	MS, UV, NMR
2	HB084, HB116, HB118, HB184	3-Formamido-2-hydroxy- <i>N</i> -[(2 <i>R</i> ,3 <i>S</i> ,6 <i>S</i> ,7 <i>R</i> ,8 <i>R</i> )-7-hydroxy-2,6,8-trimethyl-4,9-dioxo-1,5-dioxoan-3yl]benzamide (37)	Macrolide (antimycin)	ND <sup>a</sup>	MS, UV
3	HB084, HB116, HB118, HB184	{3[(3-Formamido-2-hydroxy-benzoyl)amido]-2,6-dimethyl-4,9-dioxo-1,5-dioxoan-7-yl}fomate (15)	Macrolide (antimycin)	ND	MS, UV
4	HB084, HB116, HB118, HB184	Blastamycin (37)	Macrolide (antimycin)	Antifungal inhibitor of ATP-citrate lyase	MS, UV
5	HB084, HB116, HB118, HB184	Urauchimycin A or B (46)	Macrolide (antimycin)	Antifungal	MS, UV
6	HB084, HB116, HB118, HB184	Dehexylantimycin A1 (15)	Macrolide (antimycin)	ND	MS, UV
7	HB084, HB095, HB096, HB116, HB118, HB184	Antimycin A3 (8)	Macrolide	Cytotoxic, antiviral, inhibitor of ATP-citrate lyase	MS, UV, NMR
8	HB084, HB095, HB096, HB116, HB118, HB184	Antimycin A2 (8)	Macrolide	Inhibitor of ATP-citrate lyase	MS, UV, NMR
9	HB084, HB095, HB096, HB116, HB118, HB184	Antimycin A1 (8)	Macrolide	Antiviral, inhibitor of ATP-citrate lyase, ichthyotoxin, inhibitor of ubiquinol-cytochrome <i>C</i> oxidoreductase	MS, UV, NMR
10	HB084, HB095, HB096, HB116, HB118	Antimycin A0 (8)	Macrolide	Antifungal	MS, UV, NMR
11	HB095, HB096	Gentamicin C1 (12)	Aminoglycoside	Antibacterial	MS, UV
12	HB095, HB096	Gentamicin A2 (12)	Aminoglycoside	Antibacterial	MS, UV
13	HB095, HB184	Antimycin A4 (8)	Macrolide	Inhibitor of ATP-citrate lyase	MS, UV, NMR
14	HB096	Dihydromaltophilin (34)	Macrolactam	Antifungal	MS, UV
15	HB105	Polyene	Polyketide	Antifungal	UV
16	HB116, HB118	Fredericamycin A (117)	Aromatic polyketide	Cytotoxic generator of free radicals	MS, UV
17	HB122	Pyrocoll (22)	Alkaloid	Antibacterial, antifungal, cytotoxic, antiprotozoan, antiparasitic	MS, UV, NMR
18	HB122	Streptazone B1 or B2 (90)	Alkaloid	Cytotoxic	MS, UV
19	HB122	Saphenyl ester D (65)	Phenazine	Antibacterial	MS, UV
20	HB122	Xanthobaccin A (80)	Macrolactam	Antifungal	MS, UV
21	HB122	Aestivophoenin C (61)	Phenazine	Neuronal cell-protecting agent, antioxidant	MS, UV
22	HB184	Enterocin (88)	Aromatic polyketide	ND	MS, UV
23	HB184	<i>trans</i> -Cinnamic acid (15)	Aromatic carboxylic acid	ND	MS, UV
24	HB184	{8-Benzyl-3-[(3-formamido-2-hydroxybenzoyl)amino]-2,6-dimethyl-4,9-dioxo-1,5-dioxonan-7-yl} 3-methylbutanoate (15)	Macrolide (antimycin)	ND	MS, UV
25	HB184	Antimycin (8)	Macrolide (antimycin)	ND	MS, UV
26	HB184	5-{3-[(3-Formamido-2-hydroxybenzoyl)amino]-2,6-dimethyl-8-octyl-4,9-dioxo-1,5-dioxonan-7-yl} pentanoic acid (37)	Macrolide (antimycin)	ND	MS, UV
27	HB241	Indole derivative	Alkaloid	ND	MS, UV

<sup>a</sup> ND, not determined.

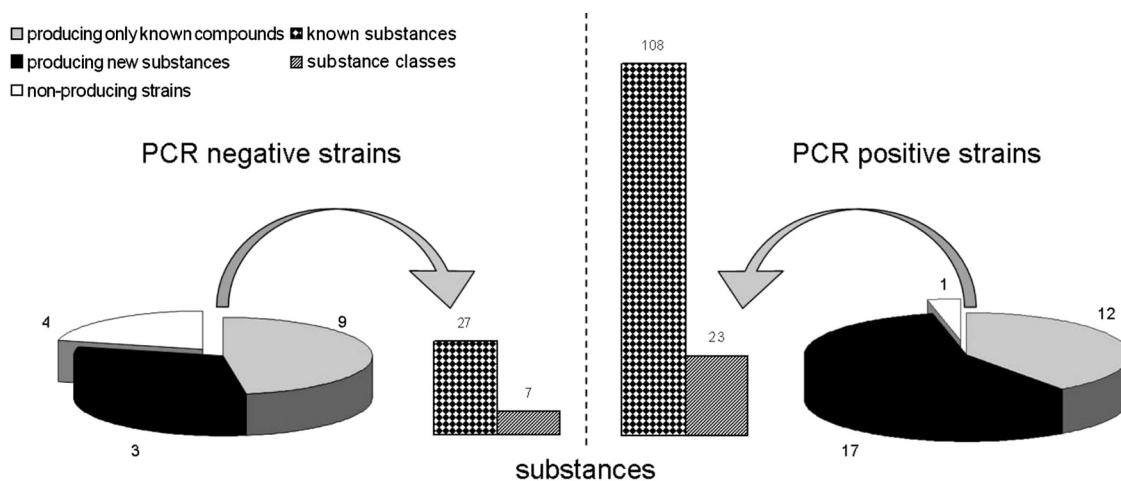


FIG. 1. Comparison of the numbers of secondary metabolite-producing strains (pie diagrams) and the numbers of resulting compounds (bars) among strains with a negative NRPS/PKS PCR result and those with a positive PCR result.

strain HB238, which seems to be a plant growth regulator, were detected.

**Antimicrobial activities of identified metabolites compared to crude extract activities.** Overall, by comparing data on the antimicrobial activities of the extracts (Table 4) with the data for identified metabolites and their known bioactivities from the literature (Tables 2 and 3), we were able to relate the antimicrobial activities of the extracts to the corresponding identified antimicrobial metabolites in 31 culture extracts (Table 4). The multiple antimicrobial activities of some extracts may be explained by the presence of antibiotics with broad spectra of activities. Another explanation may be the occurrence of many different substances with complementary activities. For example, two antibacterial metabolites and, in addition, the cytotoxic compound 4-thiouracil were identified in strain HB107 (Table 2). However, for six strains, antimicrobial activity was measurable although substances with described antibacterial or cytotoxic activities were not identified. For example, strain HB132 showed activity against different bacteria, but only two antifungal substances, venturicidin A and irumamycin, were identified (Tables 2 and 4). These findings may indicate the presence of new antibacterial compounds, an idea which is supported by the detection of a so far unidentified substance in the extract from strain HB132. To prove the correlation between antimicrobial activities of the crude extracts and the presence of secondary metabolites, strains HB100, HB157, and HB288 were selected for isolation of metabolites. The activities of the pure substances against our test organisms were examined. The antimicrobial activities of the culture extracts were congruent with those of the purified substances. For example, the extract from HB100 showed activity against *R. solanacearum*, which was attributed to the oxazolinomycin isolated from this strain. It is already known from literature that this substance shows such activity. Extracts from HB157 and HB288 inhibited the growth of *C. glabrata*, which is consistent with the presence of antimycins A0 to A4, showing the same activity patterns (Table 4).

## DISCUSSION

**Correlation between NRPS and PKS-II biosynthetic gene fragment occurrence and secondary metabolite pattern.** The majority of *Actinobacteria*-derived compounds are shown to be complex polyketides and nonribosomal peptides; nevertheless, other metabolites and other biosynthetic pathways do exist, as reflected in actinobacterial genomes (10, 44). It may be assumed that a genome with a higher number of biosynthetic gene clusters is more likely to result in a positive hit in an NRPS/PKS gene screening approach. Therefore, positive results in a PCR-based screening for NRPS and PKS-II genes not only provide evidence of the production of corresponding metabolites but also may indicate the existence of further metabolic pathways of secondary metabolite synthesis.

However, the lack of detectable NRPS or PKS-II gene fragments does not definitely prove the absence of the respective biosynthetic gene clusters. For example, the strains HB116, HB118, and HB157 produced the polyketide fredericamycin A. The biosynthetic gene cluster for this substance showed an unusual KS $_{\alpha}$  domain which is not amplified by the primer system used. Sequence comparison of the applied PKS-II primers and the fredericamycin A gene cluster did not reveal any homology. Hence, the chosen primer system, albeit favorable for the great majority of known PKS-II genes, is not working in the case of aromatic polyketides with unusual molecular constructions. Within this study, all bacteria—except a single isolate—that tested positive in the genetic prescreening approach were not only able to produce a higher number of secondary metabolites but also provided greater diversity of structural types and more antimicrobial activity than those strains with a negative result for PCR amplification of PKS-II and NRPS genes. Furthermore, the number of strains that did not produce any compound under the applied culture conditions was also significantly higher within the group with a negative result. For this reason, molecular screening for PKS and NRPS genes is a valuable tool for preselection of strains for secondary metabolite production.



TABLE 4. Antimicrobial activities of extracts from actinobacterial strains<sup>a</sup>

Strain	Active against:									Antimicrobial activity established by:	
	<i>S. lentus</i>	<i>B. subtilis</i>	<i>E. coli</i>	<i>P. fluorescens</i>	<i>C. glabrata</i>	<i>P. syringae</i>	<i>X. campestris</i>	<i>E. amylovora</i>	<i>R. solanacearum</i>	Testing of extracts	Description of identified compounds in the literature
HB062	X									X	X
HB084		X								X	X
HB094											
HB095		X								X	X
HB096		X								X	X
HB099											
HB100									X <sup>b</sup>	X	
HB101											
HB102											X
HB105		X								X	X
HB107		X	X	X	X	X	X		X	X	X
HB108											
HB110											
HB113											X
HB114											
HB115											
HB116		X								X	X
HB117	X	X			X				X	X	X
HB118		X								X	X
HB122	X	X								X	X
HB132	X	X	X	X	X		X	X		X	X
HB138	X	X				X	X			X	X
HB140											X
HB142					X				X	X	
HB149	X				X					X	
HB156					X				X	X	X
HB157					X <sup>b</sup>					X	X
HB181		X								X	X
HB184	X	X								X	X
HB200											
HB202	X	X		X			X	X		X	X
HB238									X	X	
HB241											
HB243											X
HB253	X	X	X		X		X		X	X	
HB254									X	X	
HB272	X	X				X			X	X	X
HB274					X				X	X	X
HB288	X <sup>b</sup>				X <sup>b</sup>					X	X
HB291							X			X	X
HB298	X	X	X		X	X	X	X		X	X
HB320											
HB328	X	X						X		X	X
HB375	X	X			X				X	X	X
HB381											
HB383	X	X								X	
No. of extracts with activity	15	20	4	3	12	4	7	4	11	31	29
% of extracts with activity	33	44	9	7	26	9	15	9	24	67	63

<sup>a</sup> X indicates positivity for antimicrobial activity.<sup>b</sup> Correlation between the biological assay results for crude extracts and those for purified substances was possible.

**Ecological roles of compounds: beneficial effects.** Though the abundance of microorganisms in marine sponges is well examined, the ecological roles of secondary metabolites produced by these microorganisms are not well understood. It was hypothesized that they may be relevant in biological interactions, e.g., in defense against antagonists. In the following, the activities of the compounds identified in the extracts from

sponge-associated *Actinobacteria* will be discussed from an ecological point of view.

In the close bacterium-sponge associations, *Actinobacteria* represent a large proportion of the microbial community compared to the marine bacterioplankton (41, 47, 109). *Actinobacteria* display enormous potential to produce secondary metabolites within bacterium-invertebrate communities (16, 38, 56,

108) and thus may contribute to the survival of the organisms involved. One possible function of these compounds is the chemical defense of the host (41). In the case of a sponge, detrimental organisms range from pathogenic bacteria and fungi to macroorganisms which colonize the surface or prey on the sponge. The protection of *H. panicea* against infectious agents or predators may be mediated by the substances which were identified in this study, because they showed broad-spectrum activities, including antimicrobial and cytotoxic effects.

Antibacterial agents like the streptophenazines (produced by strain HB202) and different streptomycins (produced by strains HB062, HB113, and HB138) may defend *Actinobacteria* and their host against other bacteria (29, 74, 116, 118). Through the production of antifungal agents like pyrocoll (produced by strains HB117, HB122, and HB328), *Actinobacteria* defend themselves against fungi in their environment. Fungi are competitors for nutrients and also produce antibacterial substances. Many of the secondary metabolites identified in the *Actinobacteria* associated with *H. panicea* are cytotoxic: streptazone B1 (90) (produced by strains HB117, HB122, and HB291), fredericamycin A (117) (produced by strains HB116, HB118, and HB157), and daryamide C (7) (produced by strain HB272). These substances may be protective against different eukaryotic antagonists like protists or vertebrates. They also may serve as protective agents against digestion of the bacteria by the sponge itself. Insecticides and other agents against vertebrates and invertebrates, like antimycin A1 (8) (produced by strains HB157, HB181, and HB288) and bafilomycin (60) (produced by strain HB181), as well as streptomycin (116) (produced by strain HB113), may defend the sponge from predators. Antioxidative agents like aestivophoenin C (produced by strain HB122) may shield the sponge against the oxidation of sensitive molecules (61) and protect the cell nucleus and the cell membrane from these "scavengers." However, many redox-active secondary metabolites may have additional and relevant properties independent from their antioxidant/radical-scavenging activities. One example is a modulation of colony morphology by phenazines of *Pseudomonas aeruginosa* (23), but antibiotic activities and cell-cell-signaling functions have been reported as well. Therefore, antioxidant agents may also play a role in the regulation of growth and the composition of biofilms in *H. panicea*. Phytotoxic compounds and algicides may prevent the sponge from periphyton algae (53, 104). An example of such a substance is oxazolomycin (produced by strain HB100). Pyrocoll (produced by strains HB117, HB122, and HB328) and other antiprotozoan agents such as antibiotic X 14931A (produced by strain HB113) may protect the bacteria against digestion by protozoans (22). In summary, the substances produced by *Actinobacteria* possibly could participate in maintaining the balance of microbial biofilms within and on the sponge by preventing growth of deleterious microorganisms.

**Ecological roles of compounds: deleterious effects.** Beside beneficial interactions, some of the produced substances may exhibit deleterious effects on the sponge. For example, strain HB181 synthesized bafilomycin D, which inhibits ATPase (60). Analyses of the strains HB117, HB122, HB181, HB291, and HB328 revealed the presence of the cytotoxic compounds senacarin A (79) and pyrocoll (22). All these activities may affect not only potential predators but also the sponge. Known

potential virulence factors in sponges are produced by fungi, viruses, cyanobacteria, and bacteria of the genera *Bacillus* and *Pseudomonas* (119). In one case, a novel alphaproteobacterium was identified as the primary pathogen (119). Up to now, *Actinobacteria* have not been described as sponge pathogens, and we assume that the secondary metabolites produced by *Actinobacteria* associated with *H. panicea* rather have beneficial effects.

**Biological sources of the substances.** An increasing number of compounds originally thought to be biosynthesized by the sponges are actually produced by sponge-associated bacteria (89), e.g., the peptide thiocoraline, produced by a *Micromonospora* species (97). This inevitably raises the question of whether sponge-associated *Actinobacteria* provide a number of metabolites whose production has been attributed to sponges before. Nearly all compounds from *Actinobacteria* strains isolated from *H. panicea* that we have identified within our study were known as bacterial products. In only two cases are the reported results ambiguous: 1-epimanzamine D, which was originally isolated from a Palaun sponge and was produced by strain HB113, identified as *Streptomyces fulvorobeus* (127), and coproporphyrin, produced by strain HB100 (113), were described previously as products of sponges (coproporphyrin was also identified as a product of bacteria). It remains unknown whether all of the substances produced by *Actinobacteria* in laboratory cultures are also synthesized in the sponge habitat, what factors are essential for production, and whether the compounds will be produced in sufficient amounts to interact with the environment. Here, it is important that microbial secondary metabolites have been proposed to act as signaling molecules at subinhibitory concentrations. Thus, it is not expected that these compounds are produced at inhibitory levels. So far, some bacterium-derived substances have been isolated from sponge tissue (55, 87, 89), which may be taken as evidence that their concentrations are sufficient to be biologically active *in situ*. A specific cocktail of chemical substances that is strongly dependent on environmental factors is expected to occur. Hence, only a small selected fraction of the secondary metabolites of *Actinobacteria* will be expressed at a given time. Within this context, it is of special interest that the first chemical analysis of crude extract from *H. panicea* revealed the occurrence of antimicrobially active substances identified in the sponge-associated *Micromonospora* sp. strain HB243. In conclusion, some of the substances identified in sponge-associated *Actinobacteria* are definitely produced within the sponge in sufficient amounts to display their biological activities. Depending on the environmental conditions, we expect that most of the other compounds described in this work, if not all, may have their roles in the complex sponge system.

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